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(54) Method for measuring cholinesterase and method for distinguishing between liver cirrhosis and hepatitis

(57) The object of the present invention is to provide a method for discriminating between liver cirrhosis and chronic hepatitis by serodiagnosis using a simple enzyme immunoassay method without performing histopathological examinations by biopsy and so forth.

The present invention discloses an enzyme immunoassay method for cholinesterase using a monoclonal antibody, a method for detecting Aleuria aurantia lectin-reactive cholinesterase using a monoclonal antibody and Aleuria aurantia lectin, and a method for discriminating between liver cirrhosis and chronic hepatitis on the basis of those results.

EP 0 738 890 A1

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Description

The present invention relates to a method for measuring total cholinesterase in, for example, serum, a method for measuring Aleuria aurantia lectin-reactive cholinesterase in, for example, serum, and an in vitro diagnostic method for distinguishing liver cirrhosis, liver carcinoma and hepatitis using those methods.

Two types of cholinesterase exist in the body that differ in terms of enzymological properties, physiological function and distribution in the body. Namely, the first is acetylcholinesterase (E.C.3.1.1.7), which specifically breaks down acetylcholine, exists in a large amount in erythrocytes, neural tissue, muscle and so forth, and is distributed in relation to these physiological functions. The other is cholinesterase (also referred to as pseudocholinesterase or butylcholinesterase) (E.C.3.1.1.8), which acts on cholines such as benzoylcholine and butylcholine, exists in a large amount in the serum and liver, is produced in the liver, and the physiological action of which is considered to most likely be involved with the neuromuscular system.

At present, the cholinesterase that is frequently measured in clinical laboratory examinations is cholinesterase in serum (pseudocholinesterase or butylcholinesterase). This enzyme is a glycoprotein having a molecular weight of approximately 340,000 and is composed of four identical subunits. Each subunit is composed of 574 amino acids, and has nine asparagine-coupled carbohydrate chains. Clinically, a decrease in its activity, as determined by measuring this enzyme, has significance in terms of determining the degree of functional impairment of liver parenchyma in liver disease, and particularly chronic liver parenchymal disorders such as liver cirrhosis and chronic hepatitis. Since serum cholinesterase is produced in the liver parenchymal cells, its decrease indicates a chronic functional decrease of liver cells. In addition, acute decreases in cholinesterase activity are observed in cases of poisoning by organic phosphorous-based agricultural chemicals, and measurement of the activity of this enzyme is indispensable in these cases. In addition, increases in the activity of this enzyme are observed prominently in nephrotic syndromes.

In the past, measurement of cholinesterase was performed by various methods including a thiocholine method wherein thiocholine released by cholinesterase is measured by coloring it with an SH group assay reagent using the synthetic substrates of acetylthiocholine, propionylthiocholine and butylthiocholine, a UV method wherein a direct decrease in substrate is measured in the form of a reduction in absorbance of the ultraviolet using benzylcholine for the substrate, a pH colorimetric method wherein an organic acid produced by cholinesterase is measured using a pH indicator, and an enzyme method (cholinoxidase method) wherein the hydrogen peroxide produced during specific decomposition of choline by cholinesterase is measured with a coloration system using benzoylcholine for the substrate and cholinoxidase and peroxidase as cooperative enzymes.

In addition, ever since the development of technology for producing monoclonal antibodies by G. Köhler and C. Milstein in 1975 (Nature, Vol. 256, p. 495, 1975), numerous monoclonal antibodies have been prepared for various antigens. The use of monoclonal antibodies has proceeded in the fields of in vitro diagnostic drugs, in vivo diagnostic drugs, therapeutic drugs and affinity purification reagents. Some of these have already reached the level of practical application, while research and development are being actively conducted, on others, to reach practical application.

Monoclonal antibodies to cholinesterase have also been produced. Examples of methods that have already been reported include a method wherein cholinesterase is measured by an enzyme immunoassay method using immobilized polyclonal antibody to cholinesterase and monoclonal antibody to cholinesterase (A. Broch, et al., J. Clin. Chem. Clin. Biochem., Vol. 28, p. 222, 1990), and a method wherein cholinesterase is measured by an enzyme immunoassay method using immobilized monoclonal antibody to cholinesterase and polyclonal antibody to cholinesterase (M. Whittaker, et al., Hum. Hered, Vol. 40, p. 153, 1990).

Aleuria aurantia lectin is a protein that is free of carbohydrate chains prepared from Aleuria aurantia. It contains large amounts of serine and glycine, has a molecular weight of 72,000 and has suburits having a molecular weight of 31,000. It has one carbohydrate binding site per subunit. It has affinity for L-fucose as a monosaccharide and binds with the L-fucose $\alpha 1 \rightarrow 2$ and L-fucose $\alpha 1 \rightarrow 3$ residues, and it binds strongly with the carbohydrate chain having the L-fucose $\alpha 1 \rightarrow 6$ residue.

Recently, a serum was treated by chromatography using an affinity column on which Aleuria aurantia was immobilized (T. Ohkura and T. Hada et al.) When this column was used to measure the enzyme activity of Aleuria aurantia lectin-reactive cholinesterase retained on the column and to determine the ratio of that cholinesterase to the total enzyme activity of cholinesterase originally present in the serum, that ratio was indicated as being significantly higher in patients with hepatocellular carcinoma and liver cirrhosis than in patients with chronic hepatitis and normal controls (Cancer Research, Vol. 54, p. 55, 1994). In addition, there is no other method at present for diagnosing liver cirrhosis and chronic hepatitis than by performing histological examinations of liver tissue specimens obtained by biopsy or operation, thus causing a considerable burden on patients.

Methods for measuring cholinesterase of the prior art, including the thiocholine method, UV method, pH colorimetric method and enzyme method (cholinoxidase method), all measure the enzyme activity of cholinesterase, and do not measure the amount of cholinesterase as protein.

In addition, in the case of methods that measure enzyme activity, depending on the selection of the sub-

strate, there is the possibility of also measuring the activity of substances (enzymes) other than cholinesterase that have esterase activity. Since that which appears as a result of damage to liver parenchymal cells is inherently the synthesis of cholinesterase itself, it is necessary to measure the amount of cholinesterase as protein. In addition, even in the case of a decrease in cholinesterase activity caused by poisoning by organic phosphorous-based agricultural chemicals, measuring the amount of cholinesterase as protein along with enzyme activity allows a more accurate judgment to be made.

Moreover, in the case of the reported enzyme immunoassay methods, since polyclonal antibody is used either for the immobilized antibody or enzyme-labeled antibody, it is difficult to supply a uniform antibody semi-permanently.

Therefore, as a first object of the present invention, the inventors of the present invention attempted to measure the amount of cholinesterase as protein without being affected by substances (enzymes) other than cholinesterase that have esterase activity and without being affected by cholinesterase activity inhibitors, by using an enzyme immunoassay method that uses monoclonal antibodies that can be supplied semi-permanently in uniform quality without using polyclonal antibodies.

Moreover, since diagnosis of liver cirrhosis and chronic hepatitis by histological examinations using liver tissue specimens obtained by biopsy or operation as performed in the past, causes a considerable burden on patients, a method that enables this to be performed by serodiagnosis would be extremely significant. However, in the case of the method for determining the ratio of Aleuria aurantia lectin-reactive cholinesterase to total enzyme activity of cholinesterase originally present in serum by treating serum with column chromatography using an affinity column on which Aleuria aurantia lectin is immobilized and measuring the enzyme activity of Aleuria aurantia lectin-reactive cholinesterase retained on the column as reported in the literature since this method involves column chromatography, it becomes necessary to repeat a complex procedure consisting of adsorption, elution, measurement of volume and measurement of activity, thus making it difficult to measure a large number of samples in a short time.

Therefore, as a second object of the present invention, the inventors of the present invention attempted to develop a method for simultaneously measuring a large number of samples for Aleuria aurantia lectin-reactive cholinesterase by a simple procedure and without using column chromatography.

The present invention provides a method for measuring cholinesterase comprising the steps of:

reacting an immobilized first monoclonal antibody that recognizes cholinesterase, a labeled second monoclonal antibody that recognizes an epitope of cholinesterase different from the epitope recognized by said first monoclonal antibody and a sample, and

detecting the label of said reacted or unreacted second monoclonal antibody.

Moreover, the present invention provides a method for measuring Aleuria aurantia lectin-reactive cholinesterase comprising the step of:

reacting an immobilized monoclonal antibody that recognizes cholinesterase, a labeled Aleuria aurantia lectin and a sample and detecting the label of said reacted or unreacted Aleuria aurantia lectin.

Moreover, the present invention provides a method for discriminating between liver cirrhosis, liver carcinoma and hepatitis which comprises the step of:

measuring Aleuria aurantia lectin-reactive cholinesterase according to the above-mentioned method.

The present invention further provides a kit for measuring cholinesterase comprising a first monoclonal antibody binding to an epitope of cholinesterase and a second monoclonal antibody binding to an epitope of the cholineasterase different from the epitope recognized by the first monoclonal antibody.

The present invention also provides a kit for measuring Aleuria aurantia lectin-reactive cholinesterase comprising a first monoclonal antibody, and Aleuria aurantia lectin.

Fig. 1 is a graph showing values for Aleuria aurantia lectin-reactive cholinesterase according to disease as measured in step (1) of example 3.

Fig. 2 is a graph showing values for Aleuria aurantia lectin-reactive cholinesterase according to disease as measured in step (2) of example 3.

The following provides a detailed explanation of the present invention.

First, an explanation is provided of the first invention. The inventors of the present invention prepared a plurality of monoclonal antibodies specific for cholinesterase, and then examined immunological measurement methods using those monoclonal antibodies.

As a result, the monoclonal antibody relating to the present invention was found to be extremely useful as a reagent for cholinesterase measurement.

The monoclonal antibody in the present invention can be produced by (1) obtaining a hybridoma that produces monoclonal antibody that recognizes cholinesterase by fusing myeloma cells with mouse antibody-producing lymphocytes immunized with cholinesterase prepared from human serum, (2) culturing said hybridoma or a cell line originating from said hybridoma, and (3) collecting said monoclonal antibody from that culture. Hybridomas that produce a monoclonal antibody

that recognizes cholinesterase can be produced by cell fusion methods that are themselves known.

In the present invention, a plurality of monoclonal antibodies that recognize different epitopes of said cholinesterase are obtained by using the method described above. Since cholinesterase is a tetramer composed of four identical subunits, although it is possible to measure it using a solid phase enzyme immunoassay method according to the sandwich method using a single monoclonal antibody, since there is the possibility of competition occurring between a first antibody and a second antibody, in the present invention, measurement is carried out using a solid phase enzyme immunoassay method according to the sandwich method using two types of monoclonal antibodies recognizing different epitopes.

In the present invention, a monoclonal antibody that recognizes cholinesterase is immobilized for use as the first antibody. Known methods can be used for this immobilization method.

Examples of substances preferably used to immobilize the antibody include beads and microplates made from, for example, glass, polyethylene, polyvinyl chloride, latex, agarose, cellulose and polymethacrylate.

In addition, there are no limitations whatsoever on the labeling methods and means for labeling the second antibody, as well as on their detection methods and means, and these can be carried out using known methods and means. In methods in which an enzyme such as alkaline phosphatase, peroxidase or β-D-galactosidase is used, a radioactive substance, ^{125}I, ^{3}H or so forth is normally used for the labeling agent, while in methods in which a fluorescent substance is used, fluorescein isocyanate and so forth are normally used for the labeling agent. However, other substances may also be used.

In the case where the labeling agent is an enzyme, a substrate is used to measure its activity. Examples of substrates of alkaline phosphatase include p-nitrophenylphosphate and 4-methylumbelliferylphosphate, examples of substrates of horseradish peroxidase include 2,2'-azinodi-[3-ethylbenzothiazolinesulfonate]-2-ammonium salt (abbreviated as ABTS)-H_2O_2, 5-aminosalicylate-H_2O_2 and o-phenylenediamine-H_2O_2, and examples of substrates of β-D-galactosidase include o-nitrophenol-β-D-galactopyranoside. Known reagents such as diluents, washes, reaction stoppers and so forth are used in addition to these substrates for measurement purposes.

In the present invention, there are no particular limitations on the order of reaction of the first monoclonal antibody, the second monoclonal antibody and the sample. They may be reacted simultaneously, or reacted sequentially in any arbitrary order.

In the method of the present invention, cholinesterase in a sample is preferably measured particularly within a range of 6.25 to 600 ng/ml.

Next, the following provides an explanation of the second invention. In this second invention, although a monoclonal antibody that recognizes cholinesterase is immobilized, a monoclonal antibody the same as or similar to the monoclonal antibody of the above-mentioned first invention can be used for this monoclonal antibody. In general, in the case of obtaining a monoclonal antibody from a hybridoma or a culture of animal cells originating from said hybridoma, since the monoclonal antibody has an Aleuria aurantia lectin-reactive carbohydrate chain at the Fc region of the antibody, it is necessary to eliminate this region. For example the Fc region may be removed by performing limited hydrolysis using a protease such as pepsin or papain. As a result a $F(ab')_2$ fragment may be obtained. Alternatively, in a medium a carbohydrate chain-free monoclonal antibody can be obtained by culturing hybridoma or animal cells originating from said hybridoma, to which a carbohydrate chain synthesis inhibitor is added. Alternatively, a monoclonal antibody may be treated with carbohydrate chain decomposing enzyme. Immobilization of the monoclonal antibody from which the carbohydrate chain has been removed should be performed in the same manner as immobilization of the first monoclonal antibody of the above-mentioned first invention.

In addition, there are also no limitations whatsoever on the labeling method and means for labeling the Aleuria aurantia lectin, or on its detection method and means. Although these can be performed using known methods and means in the same manner as labeling of the second monoclonal antibody of the above-mentioned first invention, in the case the labeling agent is an enzyme, if the enzyme itself has a carbohydrate chain that binds with Aleuria aurantia lectin, it is necessary to eliminate affinity of the enzyme to Aleuria aurantia lectin by removing the carbohydrate chain.

There are no particular limitations on the reaction sequence of the monoclonal antibody, Aleuria aurantia lectin and the sample in the present invention. They may be reacted simultaneously or reacted in any arbitrary order. However, allowing the monoclonal antibody and the sample to react first followed by reaction of Aleuria aurantia lectin after removing unreacted sample is preferable since the effects of substances that bond Aleuria aurantia lectin other than cholinesterase in the sample can be removed.

In the measurement method according to the present invention, Aleuria aurantia lectin-reactive cholinesterase is measured by measuring L-fucose α1→2, α1→3 and α1→6 residues present in the carbohydrate chain of cholinesterase instead of measuring the amount of Aleuria aurantia lectin-reactive cholinesterase as protein.

A sample without dilution or a suitably diluted sample is used in the reaction in the method for detection of Aleuria aurantia lectin-reactive cholinesterase according to the present invention. In addition, measuring after diluting the sample so that the total cholinesterase concentration in the sample reaches a specific value is preferable since values are indicated that reflect the amounts of the L-fucose α1→2, α1→3 and α1→6 resi-

dues present in the carbohydrate chain of cholinesterase with respect to the total cholinesterase when comparing a plurality of samples.

In the method of the present invention, since it is difficult to measure the absolute amount of Aleuria aurantia lectin-reactive cholinesterase, the amount should be expressed as a relative value using for a reference standard samples for which the content of Aleuria aurantia lectin-reactive cholinesterase is known.

Next, the following provides an explanation of a third invention. The third invention allows discrimination of liver cirrhosis and chronic hepatitis on the basis of results obtained in the second invention of the present application. As was described in the second invention, although the sample can be used for measurement either without dilution or with suitable dilution, a dilution within 1/20 is preferable in the case the sample is serum. Measurement is then performed according to the method indicated in the second invention.

In comparison with the value of Aleuria aurantia lectin-reactive cholinesterase in the serum of normal controls, the value in hepatitis patients is roughly equal, while that of liver cirrhosis patients is significantly higher, thus enabling differentiation between hepatitis and liver cirrhosis. In addition, high values have also been observed in patients with liver carcinoma.

In addition, it is possible to discriminate between hepatitis and liver cirrhosis by comparing the measurement of Aleuria aurantia lectin-reactive cholinesterase in the serum of a subject obtained by the method in the second invention with the total cholinesterase concentration in the serum of the same subject. In this case, total cholinesterase concentration is measured separately according to the method of the first invention of the present application or a known method, and then determined by calculation. Alternatively, after measuring total cholinesterase concentration in the sample in advance and then diluting the sample so that total cholinesterase concentration in the sample reaches a specific value, preferably 0.1 to 0.5 µg/ml, the measurement according to the second invention of the present application may be performed. In this method, chronic hepatitis and liver cirrhosis can be differentiated by the amount of L-fucose $\alpha1\rightarrow2$, $\alpha1\rightarrow3$ and $\alpha1\rightarrow6$ residues present in the carbohydrate chain of cholinesterase with respect to total cholinesterase.

The present first invention is a specific measurement method for cholinesterase, and is not affected in any way by the presence of enzyme reaction inhibitors, and so forth, of cholinesterase. In addition, as the monoclonal antibody used in the present invention can be obtained in a large quantity and with uniform quality, the reaction has a high degree of uniformity. It can also be produced industrially.

In addition, the detection method of Aleuria aurantia lectin-reactive cholinesterase of the present invention is a simple method that resembles a so-called solid phase enzyme immunoassay, and does not require a complex procedure such as that of affinity chromatography. This method is effective in measuring a large number of samples.

Moreover, the hepatitis and liver cirrhosis discrimination method of the present invention is extremely significant since it provides a serodiagnosis while not causing an excessive burden on the patient since it is not performed by a histopathological examination of liver tissue specimens obtained by biopsy or operation as has been performed in the past.

In a further embodiment the invention relates to a kit for performing the above-mentioned methods. The kit comprises the embodiments outlined in the claims.

EXAMPLES

The following provides a description of the present invention through its examples. The present invention is not limited to only these examples, however.

Example 1 Preparation of Anti-cholinesterase Mouse Monoclonal Antibody

Monoclonal antibody to cholinesterase was prepared in accordance with the method of G. Köhler and C. Milstein. Namely, an emulsion containing a mixture of equal volumes of PBS (phosphate-buffered saline) containing 500 µg/ml of cholinesterase derived from human serum and Freund's complete adjuvant was intraperitoneally administered twice in 200 µl aliquots each into a BALB/c mouse at an interval of 4 weeks. After confirming the increase in antibody titer in the blood by solid phase enzyme immunoassay by detecting with horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin antibody using a 96-well plate on which cholinesterase was immobilized in 100 µl of PBS, in which 200 µg/ml of cholinesterase was dissolved, cholinesterase was intraperitoneally injected into said mouse.

Three days later, the spleen of the mouse was excised, and cell fusion was performed with 8-azaguanine-resistant myeloma cells SP2/0-Ag14 using polyethylene glycol. The cells were cultured in 96-well plates and known HAT selection was performed. Hybridoma screening was performed by solid phase enzyme immunoassay using 96-well plates, and hybridoma that produced antibody to cholinesterase was cloned by a limiting dilution method.

A plurality of hybridomas that recognize cholinesterase were obtained in the manner described above. After flask-culturing of the resulting hybridomas, they were intraperitoneally transplanted into BALB/c mice to obtain ascites containing monoclonal antibody. The antibody was then purified by ammonium sulfate precipitation and high-performance liquid chromatography using TSKgel Phenyl-5PW (TOSOH CORPORATION) to obtain a purified monoclonal antibody to cholinesterase.

In addition, after performing limited hydrolysis using pepsin, the monoclonal antibody (B-3) used for immobi-

lization in the following example was purified by ammonium sulfate precipitation and high-performance liquid chromatography using TSKgel Phenyl-5PW (TOSOH CORPORATION) and used as the $F(ab')_2$ fragment.

Example 2 Measurement of Total Cholinesterase by Enzyme Immunoassay

(1) Preparation of Immobilized Antibody

100 µl of the $F(ab')_2$ fragment of the monoclonal antibody that recognizes cholinesterase (B-3) prepared in Example 1 dissolved at 5 µg/ml in PBS was added to each well of an untreated 96-well microtiter plate (Maxisorp, NUNC), and incubated at 37°C for 3 hours. Next, the solution of each well was removed, each well was washed twice with PBS followed by the addition of 300 µl of PBS containing 1% BSA (bovine serum albumin) to perform blocking for 16 hours at 4°C (treatment in which BSA is adsorbed at non-specific binding sites with the antigen or the antibody on the support). The plate was then stored at 4°C.

(2) Preparation of Enzyme-Labeled Antibody

0.1 ml of a 1% ethanol solution of 1-fluoro-2,4-dinitrobenzene was added to 1 ml of horseradish peroxidase (HRPO) solution dissolved in 0.3 M sodium bicarbonate buffer (pH 8.1) (0.5 mg/ml), and allowed to react for 1 hour at room temperature. Next, 1.0 ml of 0.06 M sodium periodate was added and allowed to react for 30 minutes. After removing unreacted sodium periodate by addition of 1.0 ml of 0.16 M ethylene glycol, the solution was dialyzed against 0.01 M sodium bicarbonate buffer (pH 9.5).

Next, 5 mg of monoclonal antibody (A-2) recognizing an epitope of the antigen different from that recognized by B-3 was added and allowed to react for 5 hours. 5 mg of sodium borohydride was added after which the solution was allowed to stand for 16 hours at 4°C.

The reaction product obtained in this manner was then purified by high-performance liquid chromatography using TSKgel G3000SW (TOSOH CORPORATION) to obtain HRPO-labeled monoclonal antibody.

(3) Measurement of Total Cholinesterase by Enzyme Immunoassay

After returning the microtiter plate, on which antibody was immobilized in step (1) of the present Example, to room temperature and washing twice with PBS, 100 µl aliquots of PBS containing 1% BSA containing 6.25 to 600 ng/ml of purified cholinesterase was added to each well as standard (the concentration of a solution of purified cholinesterase that exhibits an absorbance of 1 at 280 nm was taken to be 1 mg/ml). After incubating for 2 hours at 25°C and removing the solution, the plate was washed 5 times with PBS containing 0.05% Tween-20 (PBS-T). Next, 100 µl aliquots of PBS-T solution containing 0.1% BSA, in which the HRPO-labeled antibody prepared in step (2) was dissolved to a concentration of 4.5 µg/ml, was added to each well. After incubating for 1 hour at 37°C, the solution was removed and the plate was washed 3 times with PBS-T. Moreover, 100 µl aliquots of substrate solution consisting of 0.1 M citrate buffer (pH 4.1) containing 0.6 mg/ml ABTS and 0.01% H_2O_2 was added to each well. After allowing to react for 15 minutes at room temperature, 100 µl aliquots of 0.2 M oxalic acid were added to each well to stop the reaction.

After stopping the reaction, absorbance was measured for each well by an automated microtiter plate reader at a measuring wavelength of 415 nm and reference wavelength of 600 nm. The result shown in Table 1 was obtained from the concentration and absorbance of the standard.

Table 1

Cholinesterase Concentration (ng/ml)	Absorbance at 415 nm
0	0.077
6.25	0.089
12.5	0.100
25.0	0.131
50.0	0.175
100.0	0.273
200.0	0.480
400.0	1.020
600.0	1.592

In addition, the same procedure was performed using a 1:100 dilution of human serum diluted with PBS containing 1% BSA for the sample instead of the standard. The total amount of cholinesterase in the serum was determined by converting the concentration using the calibration curve obtained from the standard. The result is shown in Table 2.

Table 2

Sample Number	Cholinesterase Concentration (μg/ml)
1	21.4
2	28.0
3	19.0
4	16.7
5	8.9
6	13.1
7	13.7
8	18.5
9	18.7
10	22.9

Example 3 Detection of Aleuria aurantia Lectin-reactive Cholinesterase in Serum

(1) Detection of Aleuria aurantia lectin-reactive cholinesterase in samples having an equal concentration of cholinesterase

The total cholinesterase concentration in serum was measured in advance using the method of step (3) of Example 2, samples were prepared by diluting with PBS containing 10% BSA so that each serum sample had a cholinesterase concentration of 0.5 μg/ml. After returning a microtiter plate prepared in the same manner as in step (1) of example 2 to room temperature and washing twice with PBS, 100 μl of sample having an equal concentration of cholinesterase was added and incubated for 2 hours at 25°C. After removing the solution, the microtiter plate was washed 5 times with PBS containing 0.05% Tween-20 (PBS-T) and 3 times with PBS.

Next, 100 μl aliquots of PBS containing 4 μg/ml of biotin-labeled Aleuria aurantia lectin (Honen Co., Ltd.) and 4 μg/ml of streptoavidin-labeled alkaline phosphatase (Jackson Immunoresearch Laboratories Inc.) were added to each well followed by incubation for 2 hours at 25°C. After removing the solution and washing 4 times with PBS, 100 μl aliquots of 10 mM p-nitrophenylphosphate, 2 mM $MgCl_2$ and 0.3 M 2-amino-2-methyl-1-propanol were added to each well followed by incubation for 10 minutes at 25°C. After stopping the reaction by addition of 100 μl of 1 N NaOH, absorbance at a measurement wavelength of 405 nm and a reference wavelength of 492 nm was measured for each well by an automated microtiter plate reader. The result shown in Fig. 1 was obtained after classification into hepatocellular carcinoma patients, liver cirrhosis patients, chronic hepatitis patients and normal controls.

(2) Detection of Aleuria aurantia lectin-reactive cholinesterase using serum samples diluted by the same dilution factor

With the exception of using serum diluted 1:10 with PBS containing 10% BSA for the samples, and using the $F(ab')_2$ fragment of monoclonal antibody (B-3) immobilized in each well of the microtiter plate at a concentration of 3 μg/ml, detection of Aleuria aurantia lectin-reactive cholinesterase was performed using the same method as in step (1) of Example 3. The result shown in Fig. 2 was obtained after classifying into hepatocellular carcinoma patients, cirrhosis patients, chronic hepatitis patients and normal controls.

For both the result of detection of Aleuria aurantia lectin-reactive cholinesterase when serum having equal concentration of cholinesterase was used for the samples (Fig. 1), and the result of detection of Aleuria aurantia lectin-reactive cholinesterase when serum diluted by the same dilution factor was used for the samples (Fig. 2), values were low in chronic hepatitis patients and normal controls and high in liver cirrhosis patients. Thus, either method can be used to discriminate between chronic hepatitis (hepatitis) and liver cirrhosis by serodiagnosis.

Claims

1. A method for measuring cholinesterase comprising the steps of:

   reacting an immobilized first monoclonal antibody that recognizes cholinesterase, a labeled second monoclonal antibody that recognizes an epitope of cholinesterase different from the epitope recognized by said first monoclonal antibody and a sample, and
   detecting the label of said reacted or unreacted second monoclonal antibody.

2. A method for measuring Aleuria aurantia lectin-reactive cholinesterase comprising the steps of:

   reacting an immobilized monoclonal antibody that recognizes cholinesterase, a labeled Aleuria aurantia lectin and a sample, and
   detecting the label of said reacted or unreacted Aleuria aurantia lectin.

3. The method according to claim 2, wherein the monoclonal antibody is a carbohydrate chain-free antibody derivative.

4. The method according to claim 3, wherein the carbohydrate chain-free monoclonal antibody deriva-

tive is an antibody from which the Fc region is deleted.

5. The method according to claim 3, wherein the carbohydrate chain-free monoclonal antibody derivative is the $F(ab')_2$ fragment.

6. A method for discriminating between liver cirrhosis or liver carcinoma, and hepatitis comprising the step of:

measuring Aleuria aurantia lectin-reactive cholinesterase in a blood sample obtained from a subject to be tested according to the method described in claim 2.

7. The method according to claim 6, wherein the amount measurement of Aleuria aurantia lectin-reactive cholinesterase of a subject to be tested is compared with that of a healthy control.

8. The method according to claim 6, wherein the measurement of Aleuria aurantia lectin-reactive cholinesterase in the serum of a subject is compared with the total cholinesterase concentration in the serum of the same subject.

9. A kit for measuring cholinesterase comprising a first monoclonal antibody binding to an epitope of cholinesterase and a second monoclonal antibody binding to an epitope of the cholinesterase different from the epitope recognized by the first monoclonal antibody.

10. The kit according to claim 9, wherein the first monoclonal antibody is immobilized on a solid support.

11. A kit for measuring Aleuria aurantia lectin-reactive cholinesterase comprising a first monoclonal antibody binding to the cholinesterase, and Aleuria aurantia lectin.

12. The kit according to claim 11, wherein the monoclonal antibody is immobilized on a solid support.

13. The kit according to claim 11 or 12, wherein the monoclonal antibody is a monoclonal antibody derivative wherein the Fc domain is deleted.

14. The kit according to claim 11 or 12, wherein the monoclonal antibody is the $F(ab')_2$ fragment.

Fig.1

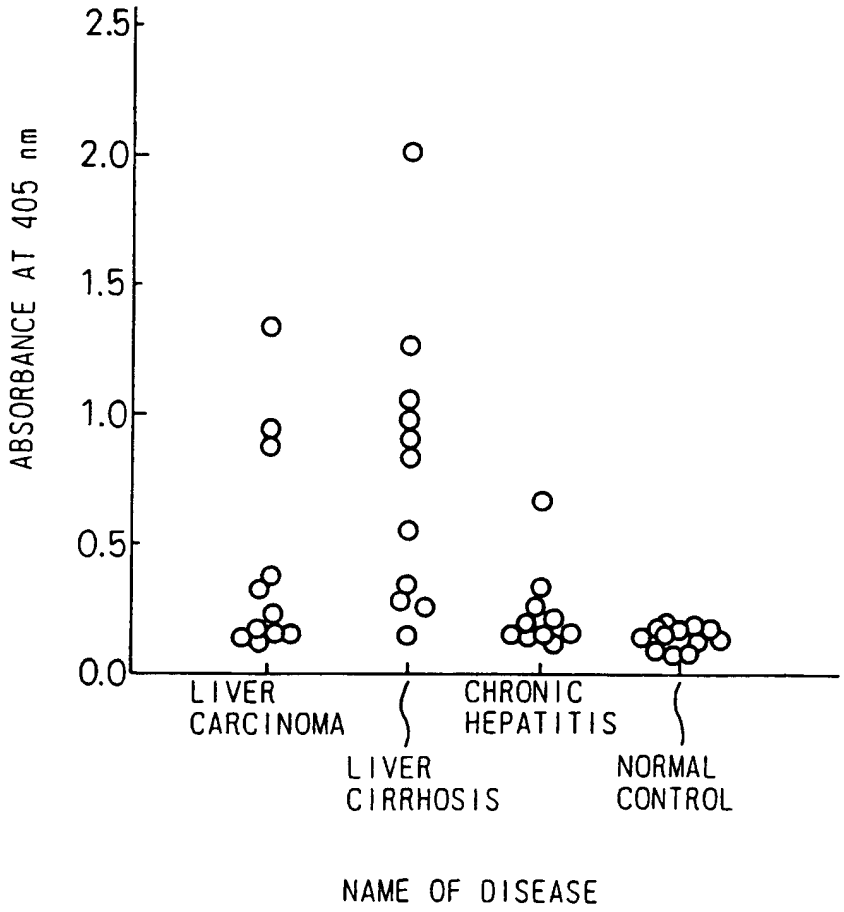
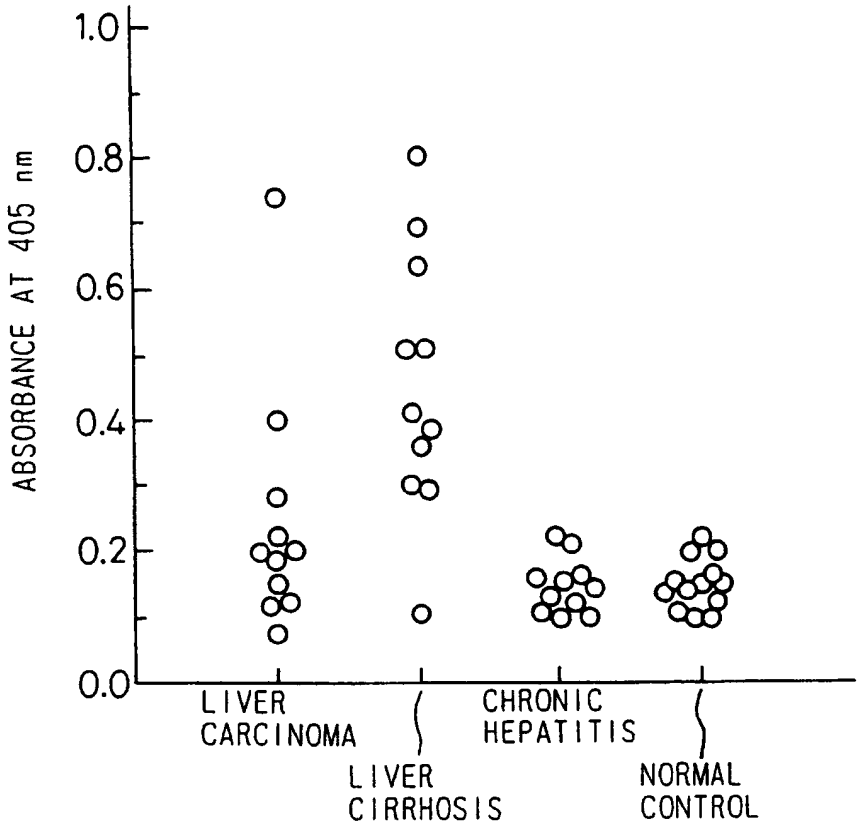


Fig. 2



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EUROPEAN SEARCH REPORT

Application Number EP 96106029.0

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
P,X	CLINICA CHIMICA ACTA, vol. 243, no. 1, December 15, 1995 M. KONDO et al. "Enzyme-linked immunosorbent assay (ELISA) for Aleuria aurantia lectin-reactive serum cholinesterase to differentiate liver cirrhosis and chronic hepatitis" pages 1-9 * Totality * --	1-14	G 01 N 33/573 G 01 N 33/577 G 01 N 33/541 G 01 N 33/566 G 01 N 33/576 G 01 N 33/563
A	CHEMICAL ABSTRACTS, vol. 118, no. 11, 1993, March 15, Columbus, Ohio, USA R.V. RAO et al. "The peptidase activity of human serum butyrylcholinesterase: Studies using monoclonal antibodies and characterization of the peptidase" page 342, no. 96 918j; & J. Protein Chem. 1993, 12(1), 103-10 --	1-8	TECHNICAL FIELDS SEARCHED (Int. Cl. 6) G 01 N 33/00
A	PATENT ABSTRACTS OF JAPAN, unexamined applications, C section, vol. 16, no. 93, March 6, 1992 THE PATENT OFFICE JAPANESE GOVERNMENT page 140 C 917; & JP-A-03 277 295 (SHIBAYAGI K.K.) --	1-8	
A	PATENT ABSTRACTS OF JAPAN, unexamined applications, C section, vol. 14, no. 566, December 17, 1990	1-8	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	03-07-1996	SCHNASS

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EUROPEAN SEARCH REPORT

Application Number

EP 96106029.0

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 6)
	THE PATENT OFFICE JAPANESE GOVERNMENT page 55 C 789; & JP-A-02 245 195 (NIPPON SHIBAYAMA HITSUJI CENTER K.K.) --		
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A	EP - A - 0 387 861 (NICHIREI CORPORATION) * Claim 9 * --	2-8	
A	EP - A - 0 441 470 (WAKO PURE CHEMICAL INDUSTRIES LIMITED) * Claims * ----	1-14	TECHNICAL FIELDS SEARCHED (Int. Cl.6)

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